Exhibit 4

REVIEW

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The color of mice: in the light of GFP-variant reporters

Abstract The mouse currently represents the premier model organism for mammalian genetic studies. Over the past decade the production of targeted and transgenic lines of mice has become commonplace, with current technology allowing the creation of mutations at base pair resolution. Such genome modifications are becoming increasingly elaborate and often incorporate gene-based reporters for tagging different cellular populations. Until recently, lacZ, the bacterial beta-galactosidase gene has been the marker of choice for most studies in the mouse. However, over the past 3 years another valuable reporter has emerged, and its attractiveness is reflected by an explosion in its use in mice. Green fluorescent protein (GFP), a novel autofluorescent genetic reporter derived from the bioluminescent jellyfish *Aequorea victoria*, currently represents a unique alternative to other gene-based reporters in that its visualization is non-invasive and so can be monitored in real-time in vitro or in vivo. It has the added advantage that it can be quantified by, for example, flow cytometry, confocal microscopy, and fluorometric assays. Several mutants of the original wild-type GFP gene that improve thermostability and fluorescence have been engineered. Enhanced GFP is one such variant, which has gained popularity for use in transgenic or targeted mice. Moreover, various GFP spectral variants have also been developed, and two of these novel color variants, enhanced yellow fluorescent protein (EYFP) and enhanced cyan fluorescent protein (ECFP), can also be used in mice. Since the spectral profiles of the ECFP and EYFP color variants are distinct and non-overlapping, these two reporters can be co-visualized, and are therefore ideal for in vivo double-labeling or fluorescent energy transfer analyses. The use of GFP and its color variants as reporters provides an unprecedented level of sophistication and represents the next step in mouse genome engineering technology by opening up the possibility of combinatorial non-invasive reporter usage within a single animal.

Keywords GFP · Transgenic mice · ES cells · Gene targeting

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The mouse as a model organism

In this article we will take green fluorescent protein (GFP) out of a cellular context and into an organismal one. We will review the current status concerning the use of GFP-based reporters in mouse targeted and transgenic regimes.

There are several reasons why the mouse has emerged as the choice model for mammalian genetic studies. In the early part of the century, mouse fanciers bred mice for their unusual traits, and thus provided the first mutants that paved the way for the introduction of this animal into the study of developmental and disease processes. The physiology of the mouse, its genome size and content mirrors that of other larger mammals including ourselves. Additionally mice are easy to breed and mutagenize.

Transgenics

A transgenic is an animal that has endogenously incorporated an exogenously applied piece of DNA into its genome. The classic, and currently most popular, route for the production of transgenics is through the introduction of DNA into zygotes by pronuclear injection.

This protocol is straightforward and has been achieved in a wide variety of animals, from livestock to laboratory mice. This method allows for the production of large numbers of transgenics, each achieved through the germline transmission of the founder animal's genome. The establishment of founder animals is so efficient that in some specific applications founders can be directly analyzed instead of using them for line establishment.

Gene targeting and embryonic stem (ES) cell technology

The advent of gene targeting and ES cell technology over a decade ago opened up new avenues for manipulating the mouse genome. ES cells are pluripotent stem cell lines derived from late blastocyst-stage embryos. They can be propagated (and manipulated) in vitro and then reintroduced into the embryo. Since the first demonstration of their ability to colonize the mouse germline, and thus have their genome transmitted to the resulting progeny, their use has become commonplace as a vehicle for germline delivery of targeted and transgenic genome modifications that are carried out in vitro.

There are two main categories of genome modification that can be carried out in ES cells, namely directed and non-directed modifications. Directed modifications are predetermined changes that are precisely engineered, and include gene targeting, whereas non-directed modifications are stochastic, and include transgenesis and mutagenesis. Both of these approaches can be incorporated into compound mutants that can be utilized in a variety of different contexts in order to provide insight into a vast array of biological questions.

Homologous and/or site-specific recombination can be used to generate any desired change to the genome. Random mutagenesis can be carried out using a variety of means, for example exogenously applied gene-trap vectors that become introduced into random locations within the genome, often resulting in "hijacked" gene expression and disruption of gene function. Another vehicle for ES cell use is for transgenesis, in a method that is analogous to the classic route by pronuclear injection. Here a vector incorporating its own promotor driving expression of a gene of interest (often a gene-based reporter) can be introduced in vitro and then taken in vivo. Thus any modification can be introduced into ES cells and then transmitted through the germline (using a chimera), so as to establish a strain of mutant mice, or studied in situ in the context of a chimera. The advantage of using ES cells over DNA injection for making a transgenic is that one will have access to an in vitro reagent in parallel with the whole animal model, since the transgenic cell line can also be propagated and studied. However as with all ES cell-mediated approaches, establishment of a transgenic line requires colonization of the germline of a chimera whereas in essence a founder animal obtained directly from an injection experiment is a heterozygote.

Chimeras

Genetic mosaics have been created and used for many years, and in many contexts they provide a powerful way to study development and disease processes in multicellular organisms (Rossant and Spence 1998). Since many mutant phenotypes are complex, it is not always possible to define the primary site of action of a given gene product, nor determine any of the later actions of that gene beyond the phenotypic "block" observed in a mutant. Thus a mosaic analysis can often help shed light on these processes. In the mouse, chimeras made by combining wild-type and mutant animals, were classically used for understanding the phenotype produced by spontaneous mutations. However, their use has recently undergone a renaissance and is reaching new levels of sophistication with novel technologies for the creation of chimeras and improved methodologies for precision genome manipulations.

When introduced into an embryonic environment either by injection into, or aggregation with, host embryos, ES cells are capable of differentiating into all derivatives of the primitive ectoderm, one of the cell layers in the blastocyst (Tanaka et al 2000). The embryonic component for the production of chimeras can either be diploid or tetraploid. Tetraploid embryos are made by electrofusion of the two blastomeres making up the embryo at the two-cell stage (Nagy et al. 1993). Tetraploid embryonic cells preferentially contribute to extraembryonic lineages in a chimera, and are excluded from the embryo proper. Thus they have a complementary contribution to that of ES cells which preferentially contribute to the embryo proper (Nagy et al. 1990).

Chimeras can be made by one of four common combinations:

1. Aggregation of two diploid embryos
2. Aggregation of a diploid embryo and a tetraploid embryo
3. Aggregation/injection of ES cells with/into a diploid embryo
4. Aggregation/injection of ES cells with/into a tetraploid embryo

Each combination is suited to address specific types of biological questions (Rossant and Spence 1998; Nagy and Rossant 1999). It should be borne in mind that, whatever the combination chosen for the production of a chimera, the incorporation of an independent genetic marker (a reporter) to follow the fate of at least one of the two components is essential in order to distinguish mutant vs wild-type cells.

Reporters

It has long been a challenge to follow the dramatic changes in cell fate, migration, and proliferation that take place during development and disease processes. In addition to

being critical for tracking the different components of a chimera, it is also often favorable to tag gene activity in, for example, a mutant ("knock-out") animal. LacZ is a gene-based reporter that has seen frequent use in tagging mutant loci and the constituent components in chimeras (Rossant and Spence 1998). Another reporter is human placental alkaline phosphatase (hPLAP). The visualization of both lacZ and hPLAP activity requires fixation of the sample and a chromogenic enzyme-based reaction (Nagy and Rossant 1999). Fluorescent protein (FP) reporters offer several advantages over enzyme-based reporters in that their visualization is quantitative and non-invasive since it requires no treatment of the experimental sample. For many years exogenously applied vital fluorescent dyes such as DiI or DiO have proved extremely useful for fluorescent labeling (Rossant et al. 1997). However, experiments incorporating these labels confer only a short window of observation and, since they were not gene-based, they cannot be inherited and thus cannot be incorporated into targeted or transgenic regimes.

The cloning of GFP was the first example of a gene-based FP reporter that is intrinsically fluorescent (Prasher et al. 1992). Its subsequent expression and fluorescence detection in the nematode worm *Caenorhabditis elegans* (Chalfie et al. 1994) has established it as a novel genetic reporter system.

GFP – a superior FP reporter

Light is produced when energy is transferred from the calcium-activated photoprotein aequorin to GFP in the bioluminescent jellyfish *Aequorea victoria* (Morin and Hastings 1971; Ward et al. 1980). When expressed in a heterologous system and illuminated by light of the appropriate wavelength (major peak at 395 nm and a minor peak at 475 nm), GFP yields a green fluorescent emission with a peak at 509 nm. Such aequorin-independent light-stimulated GFP fluorescent emission has been shown to be species independent. The GFP chromophore consists of a cyclic tripeptide comprising Ser65-Tyr66-Gly67 (Fig. 1A), which undergoes cyclization and oxidation, and is only fluorescent when embedded within the complete GFP protein (Cody et al. 1993). GFP is fluorescent either as a monomer or as a dimer, with the ratio of monomeric vs dimeric forms depending on the concentration of protein and the environment. Since GFP activity can be monitored in a wide variety of systems, the process of fluorescence emission is believed to be autocatalytic, or to require ubiquitous cellular factors.

The principal feature that makes GFP so attractive is that it combines the appealing features of gene-based reporter usage with non-invasive detection. It therefore can be used not only to tag gene expression or promoter activity, or be engineered as a fusion protein thereby providing subcellular localization, but also reporter activity can be monitored in real-time in vitro or in situ. Additionally GFP is small (27 kDa) and functions primarily as a monomer, whereas lacZ is much larger (135 kDa) and functions as a tetramer. Thus GFP-based reporters are intrinsically better suited for the creation of protein fusions that do not *per se* perturb development by, for example, delivery into the coding region of an endogenous locus ("knock-in") in order to ablate gene function.

GFP variants – a thermostable FP color palette

From the onset wild-type GFP (wtGFP) attracted a great deal of interest as a novel and simple reporter and as a potential in vivo marker. The first example of expression of wtGFP in the nematode worm prompted its use in transgenic animals and plants, including organisms such as the fruitfly *Drosophila melanogaster* (Wang and Hazelrigg 1994), zebrafish *Danio* (Amsterdam et al. 1995), and slime mold *Dictyostelium* (Hu and Cheng 1995). However, even though successful in many contexts, heterologous expression of the reporter was not always straightforward in all applications. For example, in the plant *Arabidopsis thaliana* proper expression of wtGFP was found to be curtailed because of aberrant mRNA processing (Haseloff et al. 1997) due to the presence of a cryptic intron within the coding sequence. Additionally expression of this reporter in cells (and organisms) that require incubation at high temperatures was also found to be problematic at first. Initial investigations utilizing wtGFP in mice failed to reproduce the data obtained in *C. elegans*, primarily due to the thermolability of the protein at 37°C. This therefore hampered the first endeavors to incorporate this reporter into the field of mouse gene targeting and transgenesis.

Such difficulties prompted several screens aimed at isolating mutant forms of the wtGFP protein yielding increased fluorescence and thermotolerance (see for example, Siemering et al. 1996). As a result several wtGFP variants have been recovered, many of which harbor mutations that prevent temperature-dependent misfolding of the apoprotein. Recently several new mutants of the wtGFP gene, with altered excitation and emission spectral profiles, in addition to improved thermostability and fluorescence, have also been described (Cormack et al. 1996). These spectral variants form the basis of the GFP-based reporters available for use in mice.

At present the most widely used GFP variant is the red-shifted variant, enhanced GFP (EGFP; referred to as GFPmut1 by Cormack et al. 1996). Red-shifted variants such as EGFP contain mutations that shift the maximal excitation peak toward the red end of the spectrum at approximately 490 nm, a wavelength which encompasses the band width of commonly used filter sets and argon ion lasers incorporated into most FACS machines and confocal microscopes. EGFP contains two amino acid substitutions (Fig. 1A). When excited by blue light it fluoresces approximately 30- to 40-fold more brightly than wtGFP. Also, since the wtGFP protein, along with most of the variants including EGFP, exhibits a long half-life it is not ideal for accurately tracking reduction or loss of expression. To overcome this problem dEGFP (Li et al. 1998), a
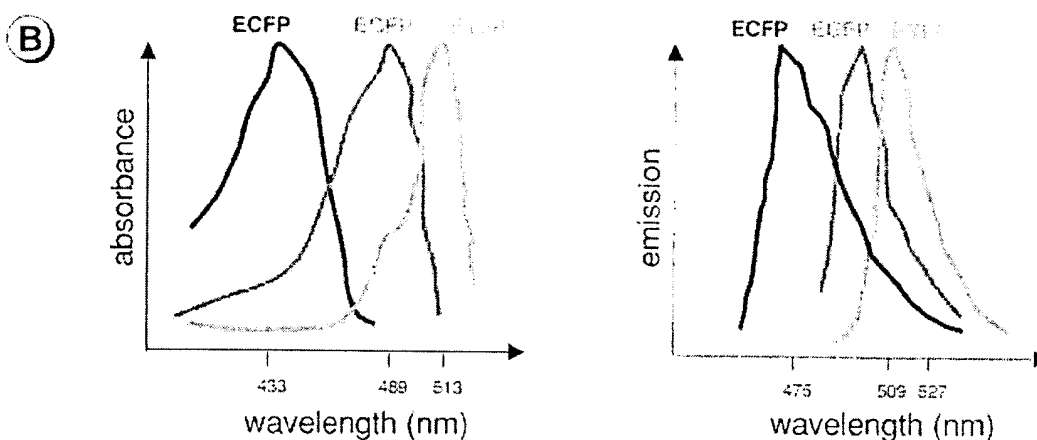
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Fig. 1A, B Enhanced cyan fluorescent protein (ECFP), enhanced green fluorescent protein (EGFP), and enhanced yellow fluorescent protein (EYFP) are three wild-type green fluorescent protein (wtGFP) variant reporters that can be used in mice. **A** wtGFP sequence in the vicinity of the tripeptide chromophore (Ser65, Tyr66, Gly67), and details of the substitutions engineered into the three variant forms of the protein. **B** Schematic representation of the excitation (*left*) and emission (*right*) spectra of these three wtGFP variants

destabilized version of EGFP, has been engineered. This was achieved by fusing the EGFP cDNA to the C-terminus of the mouse ornithine decarboxylase (ODC) gene. ODC contains a PEST sequence thereby promoting intracellular degradation of the protein. At present these destabilized variants remain untested in mice. However, because they appear not to suffer the same extended lag period for loss of fluorescence that is associated with EGFP, this class of mutants may prove useful for tracking the real-time kinetics of gene expression.

Another red-shifted variant is the enhanced yellow FP (EYFP) variant containing four amino acid substitutions (Ormo et al. 1996) that shift the emission from green to yellowish-green at 527 nm (Fig. 1B). Even though the EYFP excitation maximum lies at 513 nm it can be efficiently excited at 488 nm, a feature that poses both ad-

vantages and problems for its partnering with other variants in combinatorial regimes. The level of fluorescence of EYFP is approximately the same as that of EGFP.

At the other end of the spectrum are the blue/cyan emission variants. These include the enhanced blue FP (EBFP) variant harboring four amino acid substitutions that collectively shift the spectral profile to a shorter wavelength with excitation and emission maxima being at 380 nm and 440 nm, respectively. The introduced mutations also enhance brightness (as compared to the original blue variants) and solubility of the protein, due to improved folding and efficiency of chromophore formation (Heim and Tsien 1996). Unfortunately even though its thermostability is improved, the fluorescent signal produced by EBFP is approximately equivalent to that of wtGFP. Enhanced cyan FP (ECFP), another variant giving a blue/cyan fluorescence, does, however, produce a fluorescent signal greater than that of wtGFP. The ECFP mutant contains six amino acid substitutions, one of which shifts the emission spectrum from green to cyan, such that the excitation maximum is around 433 nm (major peak) and 453 nm (minor peak) and emission maxima at 475 nm with a small shoulder at 510 nm, as shown in Fig. 1B (Heim and Tsien 1996; Miyawaki et al. 1997). ECFP has the added advantage in that it also photo-
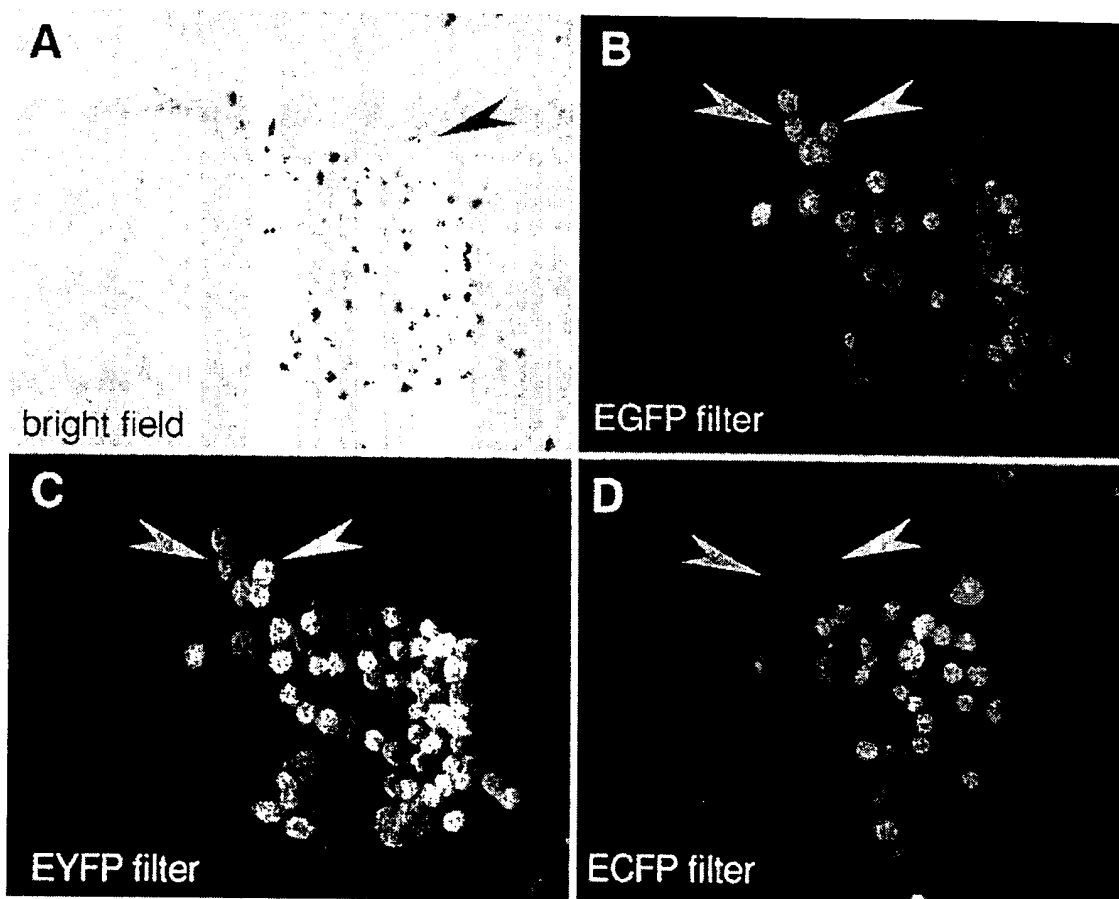
A bright field

B EGFP filter

C EYFP filter

D ECFP filter

Fig. 2A–D GFP variant expressing embryonic stem (ES) cells can be distinguished from one another using various filter combinations. Note that as the spectra for EYFP and ECFP are least overlapping, with that of EGFP falling in between both, the EGFP expression leaks into the ECFP filter band appearing a green-blue, however the EYFP does not. ECFP does not leak into the EYFP filter band, and only very slightly into the EGFP. A Bright-field view of a group of ES cells. B–D The same cells photographed through an EGFP filter set (B), EYFP filter set (C), and ECFP filter set (D). *Blue arrowhead* indicates ECFP+ cell, *green arrowhead* EGFP+ cell, and *yellow arrowhead* EYFP+ cell. Filters used for the imaging were: EGFP, excitation filter 470/40 nm, dichroic mirror 495 nm, barrier filter 500 nm long pass; ECFP, excitation filter 436/20 nm, dichroic mirror 455 nm, barrier filter 480/40 nm; EYFP, excitation filter 500/20 nm, dichroic mirror 515 nm, barrier filter 535/30 nm

bleaches less than other blue/cyan variants and is somewhat brighter than EBFP.

It should, however, be noted that at present primarily green fluorescent variants of wtGFP such as EGFP and MmGFP have been used in ES cells (Zernicka-Goetz et al. 1997; Hadjantonakis et al. 1998b) and targeted (Godwin et al. 1998) or transgenic mice (Okabe et al. 1997). However, we and others have recently investigated the other spectral variants and find that additional GFP variants are also amenable to use in mice. These will be discussed later.

Incorporation of GFP-based reporters into targeted and transgenic regimes

Ikawa and colleagues (1995) were the first investigators to report the successful use of a GFP-based FP reporter in mice. They demonstrated that rapid non-invasive selection of transgenic embryos prior to implantation was feasible with the incorporation of GFP. Another advantage of an FP reporter is that the typing of transgenics (homozygotes vs hemizygotes) is possible based on the strength of fluorescence (Hadjantonakis et al. 1998b).

Several laboratories have employed ES cells as a vehicle for transgenesis and testing the available wtGFP variants (Zernicka-Goetz et al. 1997; Hadjantonakis et al. 1998b). FP+ transgenic ES cells, as shown in Fig. 2, have been used to generate chimeras (Zernicka-Goetz et al. 1997; Hadjantonakis et al. 1998b). Chimeric embryos produced by aggregation of FP+ ES cells with morulae can be identified prior to their introduction into pseudopregnant recipient females (Hadjantonakis et al. 1998b). At present there are several strains of mice having been established by either classic injection-based (Okabe et al. 1997) or ES cell-mediated transgenesis (Hadjantonakis et al. 1998b) that exhibit widespread fluorescence initiating at the morula (eight-cell) stage through to adulthood. These reagents provide a valuable
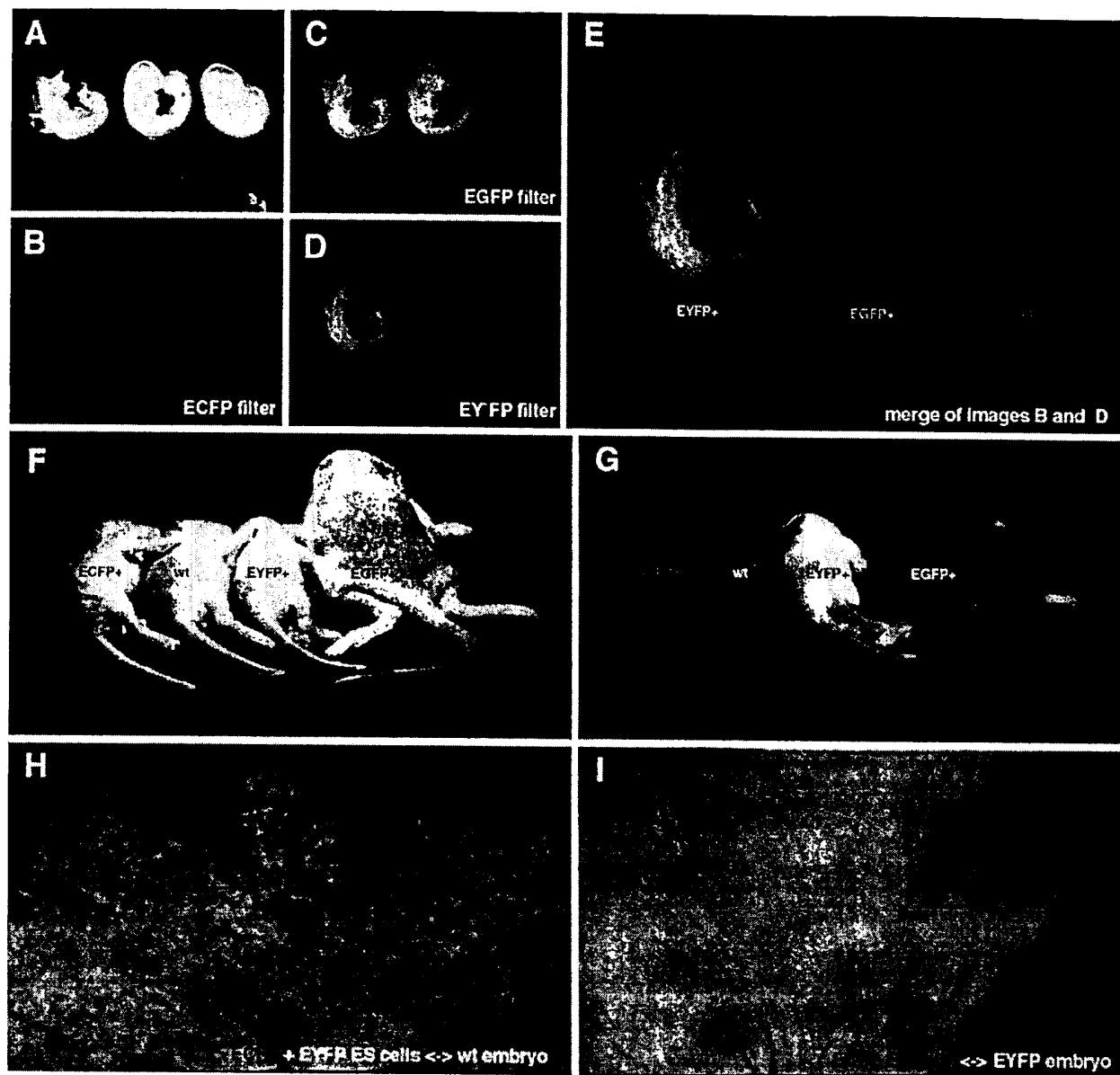
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Fig. 3A–I FP reporter expression domains can be distinguished from one another and from non-transgenics in mice by observation through the appropriate filters. The EYFP and ECFP spectra are least overlapping, with that of EGFP falling in between both. Observation of EYFP+, EGFP+, and ECFP+ mouse embryos through a variety of epifluorescence filter combinations used for excitation and capturing emission (A–E), demonstrates that ECFP and EYFP can be readily discerned, whereas the EGFP signal can leak into both fields. Transgenic EYFP+ (*left*), EGFP+ (*center*), and ECFP+ (*right*) midgestational mouse embryos (9.5 days old) photographed through bright-field (**A**), ECFP (**B**), EGFP (**C**), and EYFP (**D**) filter sets. **E** Merge of the images in **B** and **D**, and highlights the lack of overlap in the ECFP and EYFP spectra. **F** Bright-field photograph of four agouti coat-colored mouse pups. **G** Dark-field photograph taken under epifluorescence optics revealing the transgenic color of the mice. *Left to right* an ECFP+ 1-week-old pup, non-transgenic (and therefore non-fluorescent) 1-week-old pup, EYFP+ 1-week-old pup, and EGFP+ 2-week-old pup. GFP variant reporters can be individually visualized and distinguished in adult organs. Merged dark-field photographs (taken using an ECFP and an EGFP filter set) of **H**, a close-up view of the surface of a live chimeric mouse liver comprised of ECFP+, EGFP+, and non-expressing cells. **I** A close-up view of the surface of a live chimeric mouse heart comprised of ECFP+ and EYFP+ cells

source of tagged material for all stages of murine development and adulthood (Fig. 3).

There are also now an ever-increasing number of transgenic lines of mice that incorporate cis-acting regulatory elements to drive EGFP expression in a variety of different cell types and in a range of organs. One of the first published reports used the astrocyte-specific glial fibrillary acidic protein (GFAP) promoter to drive the S65T GFP variant (Zhuo et al. 1997), and compared the expression obtained with the FP reporter to that of previ-

ously established transgenic lines using the same GFAP promoter but employing lacZ as the reporter (Brenner et al 1994). This work demonstrated that the GFP and lacZ reporters produced equivalent spatiotemporal readouts when placed behind the same GFAP promoter element. Therefore GFP and lacZ could be used interchangeably. Various additional lines of transgenic mice expressing EGFP in a spatiotemporal fashion have recently been published. They include the Hoxb7 promoter which gives expression in every branch of the ureteric bud and in its derivative epithelia throughout renal development and in the adult kidney (Srinivas et al. 1999), and the Col2 promoter which serves as a reporter for the chondrocyte lineage and for chondrogenesis in live embryos and newborn pups (Grant et al. 2000). Two different laboratories have generated transgenic lines utilizing the Oct4 promoter which marks primordial germ cells (Anderson et al. 1999; Yoshimizu et al. 1999). Additionally a ubiquitously expressed EGFP reporter inserted into the X-chromosome has been used as a reagent for determining the sex of embryos 48 h after fertilization, approximately 10 days before the sex is morphologically discernible (Hadjantonakis et al. 1998a).

The first example of a gene targeting experiment that delivered an FP reporter to a specific locus was in the case of two separate murine Hox genes, Hoxa1 and Hoxc13 (Godwin et al. 1998). These two genes were chosen because one of them (Hoxa1) is expressed early and the other (Hoxc13) is expressed late during mouse embryonic development. The alleles created were "knockouts" (resulting in ablation of gene function), however, each of the targeting constructs also delivered an inframe EGFP cDNA to the targeted locus thereby also functioning as a reporter "knock-in". This study was particularly informative as it in fact described the first pair of mouse lines each harboring a single-copy EGFP cassette, therefore providing the "acid test" for the sensitivity of GFP-based reporters. Moreover, in both these alleles the EGFP reporter was translated as a fusion protein with an N-terminal portion encoded by the targeted Hox gene. Both alleles expressed the EGFP reporter thus illustrating that spatiotemporal regulation could be obtained both early in embryonic development and later into adult life. In order to further investigate the signal obtained from their EGFP alleles, the authors went on to compare the expression from their Hoxa13 EGFP "knock-in" allele (Hoxa1[GFPneo]) to the expression pattern obtained by in situ hybridization using a Hoxa13 riboprobe, and showed that it overlapped. Thus the targeted EGFP reporter was indeed functioning as a fusion protein, retaining all cis-acting elements, and lacking position effects. For the Hoxc13 EGFP "knock-in" allele (Hoxc13[GFPneo]) an equivalent lacZ-tagged allele (Hoxc13[lacZneo]) was available (Mansour et al. 1990), and thus the two reporters could be compared directly In doing so it was demonstrated that the EGFP reporter marked the same populations of cells as did the lacZ reporter, and that the sensitivity was roughly equivalent. Various other targeted lines of mice expressing EGFP in

a spatiotemporal fashion have now been reported, and include a series of mice tagged with the first fusion "knock-in" FP reporter designed to mark a subcellular compartment. Rodriguez and colleagues (1999) engineered a tauGFP fusion which they incorporated into their gene targeting constructs, and used this fusion to specifically mark axonal processes (and not the nucleus) in neurons of the vomeronasal system, a structure which mediates pheromonal effects in mammals.

The Cre/lox recombination system derived from the bacteriophage P1 currently is the preferred means for creating conditional modulations (for example, ablation or activation) of gene activity in targeted or transgenic mice (Nagy 2000). Cre/lox is a binary system requiring a Cre recombinase-expressing line (transgenic or targeted to a gene of interest) and a loxP site-containing target line (in which gene function is activated or ablated). The efficacy of the system is solely dependent on the activity of the Cre recombinase. As a consequence the need for reporter lines to monitor Cre activity has become apparent. To this end, two Cre-responsive transgenic lines have recently been established where EGFP activity is detected only after Cre-mediated excision of an intervening loxP flanked chloramphenicol acetyltransferase (CAT) gene (Kawamoto et al. 2000) or beta-geo (a beta-galactosidase neomycin phosphotransferase fusion) gene (Novak et al. 2000). Not only do such reporter lines allow the conversion of all Cre transgenic lines to EGFP lineage reporters, but in doing so open up the possibility of real-time fate mapping studies.

Novel applications of FP expressing mice – Fluorescence-activated cell sorting (FACS)

The emergence of GFP as a reporter in targeted and transgenic mouse lines has lead to the establishment and use of several novel and potentially very useful methodologies. Gaining access to cell types of interest from complex tissues would be useful for many aspects of modern biology. However, most strategies used for sorting specific cell types out from a complex population usually involve labeling cells with fluorescing antibodies, or exposing them to dyes that ultimately compromise viability. The advent of GFP-based reporters has circumvented this by allowing the refinement of protocols for isolating live, reporter-expressing cells from heterogeneous pools. When viewed through the appropriate epifluorescent optics, live FP+ cells can be observed in the context of the whole embryo or adult that is carrying a GFP reporter under the control of a spatiotemporally restricted regulatory element. In adult animal tissues or whole embryos, crude manual dissections aided by the use of enzymatic reagents, can lead to the separation of different cell layers. This can be followed by further dissociation into a suspension of single cells, which can be loaded directly onto a FACS machine. Then, the target population of cells (marked with the FP reporter) can be flow sorted. The basic scheme is illustrated in Fig. 4. In this

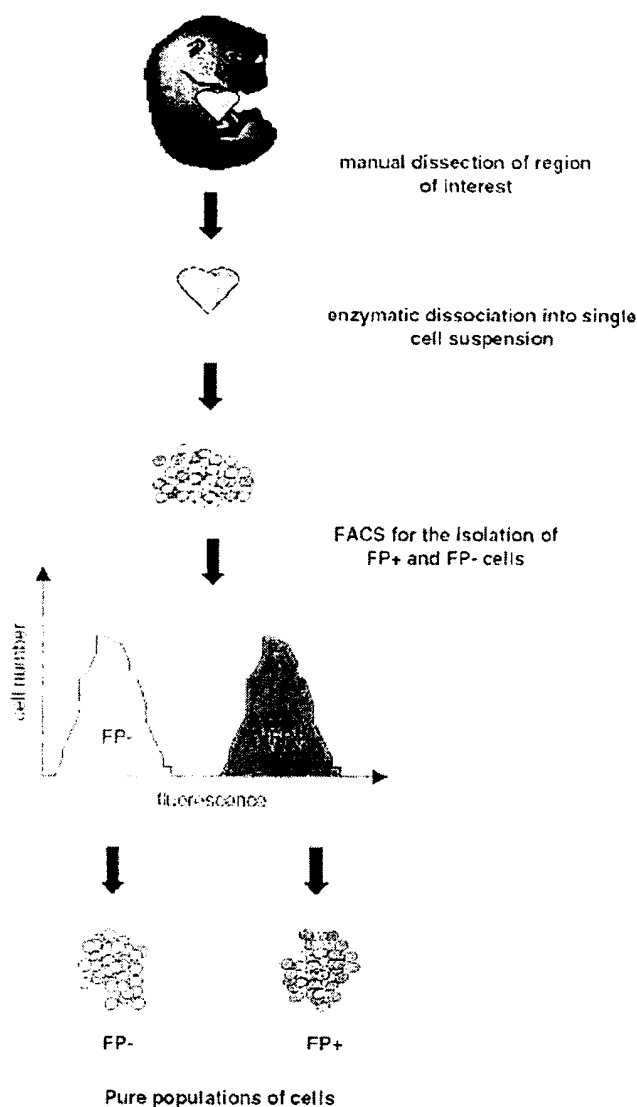an FP reporter marking cells of interest in a
transgenic or gene targeted mouse

manual dissection of region
of interest

enzymatic dissociation into single
cell suspension

FACS for the isolation of
FP+ and FP- cells

FP-

FP-   FP+

Pure populations of cells

Fig. 4 Pure populations of live FP reporter-expressing cells can be isolated from complex tissues in the embryo or adult by dissociation of the heterogeneous pool into single cells and subsequent flow sorting. Here we schematize the isolation of an FP+ population of cells in an organismal context. This involves the manual dissection and isolation of a region of interest harboring FP+ cells, the subsequent enzymatic dissociation of the complex pool in order to produce individual cells, which are then flow sorted in order to gain access to live FP+ cells which represent a population of interest

case, the transgenic embryo expresses an FP reporter in a subset of heart cells. The heart, therefore, would provide a source of material which can be triturated and subsequently flow sorted into two distinct pools, FP+ and FP-. In principle this methodology could be applied to any tissue or organ of interest.

Several reports illustrating the feasibility of this procedure have recently been published. They all demon-

strate that live FP+ cells can be obtained from mouse transgenic lines exhibiting spatiotemporally restricted EGFP expression. Pure populations of primordial germ cells (Anderson et al. 1999) and cells of the neural tube and heart (Hadjantonakis and Nagy 2000) have been recovered from midgestational embryos. Additionally, FP+ chondrocytes have been purified from the rib cages of adult mice by sorting of crude cell suspensions recovered from Col2-EGFP transgenics, and in so doing demonstrating that intensity of fluorescence correlated with biosynthesis of procollagen II (Grant et al. 2000). In another study, cells of the dermal papilla from the skin were recovered from adult mice (Kishimoto et al. 1999). The pure population of FP+ dermal papilla cells was used in a skin reconstitution assay investigating the signals required for hair follicle induction and maintenance (Kishimoto et al. 2000).

In principle this approach can be applied to recover any cell type marked with a fluorescent reporter, either from a transgenic animal expressing a spatiotemporally regulated transgene, an animal carrying a conditionally active FP reporter, chimeras generated by either aggregating FP+ ES cells with morulae (Hadjantonakis et al. 1998b) or injecting them into blastocysts (Zernicka-Goetz et al. 1997), or specialized chimeras such as those produced by injecting FP+ trophoblast stem (TS) cells into blastocysts (Tanaka et al. 1998), or homozygous "knock-out" ES cells aggregated with FP+ tetraploid embryos (Adelman et al. 2000), or even in an animal infected with an FP reporter-containing virus (Dudus et al. 1999). Such separation of individual cells from a heterogeneous pool recovered from a complex biological structure should allow the establishment of cell lines and provide access to expression-defined spatial domains, in addition to facilitating studies between equivalent cell types in wild-type vs mutant animals.

Novel applications of multi-FP expressing mice – the feasibility of non-invasive multireporter usage

Even though at present most studies using FP reporters in targeted or transgenic regimes employ the EGFP variant, this is not the only variant to be amenable to use in mice. An additional level of complexity is introduced with the use of other FP variants such as ECFP and EYFP. We have demonstrated that like EGFP both these variants can be observed as fluorescing in ES cells (Fig. 2) and mice (Fig. 3). Several lines of ES cells individually tagged with either ECFP, EGFP, or EYFP (Fig. 2; A.-K. Hadjantonakis and A. Nagy, unpublished observations) have been established and used to generate strains of mice exhibiting ubiquitous expression of each of the GFP variant reporters by germline transmission (Fig. 3A–G; A.-K. Hadjantonakis, S. Macmaster and A. Nagy, unpublished observations). Feng and colleagues (2000) have also recently incorporated multiple FP reporters in a study using transgenic mice. They reported the generation of transgenic mice where different FP
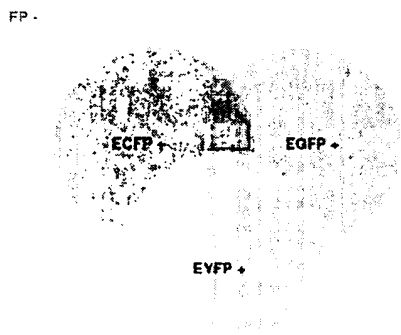
Fig. 5 The advent of wtGFP spectral variants has opened up the possibility of multi-FP reporter usage within a single animal. In this case cell populations defined by single or combinatorial reporter expression can also be imaged in a live animal or organ of interest, or flow sorted and segregated from one another in order to obtain specific populations of cells each with a signature expression profile

variants were expressed selectively in neurons. They used double transgenic mice expressing two different FPs in order to label three neuronal subsets within a single animal.

The current availability of more than one FP reporter that works in mice allows multiple recombinatorial regimes. Strategies can now be employed incorporating the use of compound transgenics each expressing different reporters, or tissue recombination or chimeric analyses where more than two compartments can be tagged, visualized, and accessed (schematized in Fig. 5). For example chimeras derived from mixed populations of both yellow (EYFP+) and cyan (ECFP+) fluorescent ES cells demonstrate that the two mutually exclusive reporter-expressing cell populations could be simultaneously visualized within a single mouse embryo or adult organ (Fig. 3H, I). Thus individual FP+ cells in single, double, or even triple transgenic animals expressing several different FPs can now be discerned.

Future applications of multi-FP expressing mice – fluorescence energy transfer (FRET)

One unique feature that the availability of spectral variants promises is for real-time analysis of protein–protein interactions in vivo using FRET, this being a non-invasive experiment aimed at the elucidation/verification of biomolecular pathways operating in a spatiotemporal context. The ECFP spectral profile makes it a suitable donor molecule in FRET studies, and so this reporter can be used for dual-labeling fluorescence microscopy in conjunction with EGFP or EYFP. To date the only GFP-based FRET experiments reported are in vitro (Mahajan et al. 1998; Nagai et al. 2000), where the utility of this approach has been highlighted by its use in interaction screens reminiscent of the yeast-2-hybrid type. However, it is only a matter of time until such a FRET experiment is carried out in live animals.

Concluding remarks

GFP is the newest gene-based reporter to be incorporated into mouse transgenic and targeted approaches. It is attractive in that it can be visualized in vivo without processing of the sample, and thus can be monitored in real-time in situ. The list of potential applications of GFP has been further extended by the isolation of mutants with altered excitation and/or emission spectra. This feature opens up the novel possibility of combinatorial non-invasive reporter usage within a single animal. Coupled with the new battery of high-resolution genome engineering approaches that can now be utilized in the study of mouse development and disease, FP reporters provide the modern day geneticist with a new and extremely useful tool.

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